Effect of *s*-triazine and phenoxyalkanoic acid herbicides on UDP-glucuronosyltransferase in rat liver microsomes

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Abstract: Twelve herbicides, representatives of two chemical groups, substituted phenoxyalkanoic acids and *s*-triazines, were tested for their inhibitory effect on the glucuronidation of 4-nitrophenol (4-NP), phenolphthalein (PPh) and 4-methylumbelliferone (4-MU) by rat liver microsomes. One millimole MCPA, ametryn and cyanazine significantly decreased PPh UDP-glucuronosyltransferase (UGT) activity, while propazine was found to be a most potent inhibitor of 4-NP glucuronidation. Concentrations of 0.1 mM dichlorprop and cyanazine were still inhibitory against PPh-UGT. The inhibition of 4-MU glucuronidation by the herbicides was low and not specific. As a whole, *s*-triazine derivatives were more inhibitory than the substituted phenoxyalkanoic acids. Kinetic studies with propazine revealed a non-competitive type of inhibition towards the acceptor substrate 4-NP, with an apparent K_i value of 0.540 mM. With ametryn, an uncompetitive type of inhibition against PPh and a mixed type of inhibition towards UDPGA were found, with apparent K_i values of 0.330 mM and 0.380 mM, respectively.

Keywords: UDP-glucuronosyltransferase; herbicides; inhibition; 4-nitrophenol; phenolphthalein; 4-methylumbelliferone

1 INTRODUCTION

The wide use of crop-protection chemicals to reduce loss due to weeds, insects and disease is an integral part of modern agriculture, so exposure to pesticides has become a serious environmental problem. These chemicals could be a source of hazard to animals and humans for their presence everywhere – in soils and in drinking water, in many natural products and foodstuffs. Therefore, the importance of providing data about biological side-effects of these substances has been generally recognized. There are many reports dealing with different aspects of pesticide effects in animals, cell cultures or enzyme systems.[1–8]

Little is known about the influence of pesticides on UDP-glucuronosyltransferases (UGT), key enzymes in a major detoxification pathway in all vertebrates. UGT are a large multigene family of membrane-bound isoenzymes, located mainly in the endoplasmic reticulum of liver and, to a lesser extent, in all other mammalian tissues. They catalyze the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to many structurally unrelated compounds which possess a hydroxyl-, carboxyl-, amino- or sulfhydryl group, converting them to water-soluble β-(D)-glucuronides. Thus, glucuronidation is involved in the biotransformation and detoxification of a diverse range of drugs, environmental chemicals, carcinogens and endogenous substances.[9–13] To date there have been identified 16 UGT isoforms in the rat, separated into two families (UGT1 and UGT2) on the basis of their sequence similarity. The known substrates of UGT1 members include bilirubin and small planar phenols, such as 1-naphthol, 4-nitrophenol and 4-methylumbelliferone.[13] At least three isoforms are known to be involved in the glucuronidation of 4-nitrophenol.[10,11] UGT2 isoenzymes conjugate bulky substrates, including endogenous molecules (steroids, bile acids) as well as morphine, phenolphthalein etc.[13,14]

In our previous work, a number of herbicides, insecticides and fungicides, belonging to different chemical groups, were tested as inhibitors of UGT.[15] Here we extend our studies on the effect of 12 representatives of two main groups of herbicides, *s*-triazines and substituted phenoxyalkanoic acids, on

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Contract/grant sponsor: Bulgarian Ministry of Education, Science and Technology; contract/grant number: B-624
(*Received 20 July 1998; revised version received 8 February 1999; accepted 24 April 1999*)

the glucuronidation of three substrates, 4-nitrophenol, phenolphthalein and 4-methylumbelliferone.

2 EXPERIMENTAL METHODS

2.1 Pesticides and chemicals

2,4-D was purchased from Aldrich Chemical Co, Milwaukee, WI, USA; MCPA, dichlorprop and 4-CPA were obtained from Sigma Chemical Co, St Louis, MO, USA; atrazine was from Serva, Heidelberg, Germany; cyanazine was from Du Pont de Nemours Co, Wilmington, DE, USA. Ametryn, prometryn, desethylatrazine, desisopropylatrazine, propazine and mecoprop were kindly donated by the Institute of Biochemistry, Martin-Luther-University, Halle, Germany. All other chemicals were purchased from Sigma.

2.2 Preparation of microsomes

Liver microsomes from male Wistar rats were isolated by differential centrifugation (10 000 *g* for 10 min and 105 000 *g* for 60 min). The microsomal pellets were suspended in Tris-HCl buffer (0.1 M; pH 7.4) containing sucrose (0.25 M), and stored at −70 °C until used.[16] The microsomal protein was determined according to Lowry *et al*[17] with bovine serum albumin as a standard.

2.3 Enzyme assay

UGT activities towards 4-nitrophenol (4-NP) or phenolphthalein (PPh) were assayed as described elsewhere.[14,18] Glucuronidation of 4-methylumbelliferone (4-MU) was assayed according to Lilienblum *et al*[19] with the modifications of Battaglia *et al*[20] using ethyl acetate instead of chloroform for the extraction of the unconjugated substrate. The standard incubation mixture in a final volume of 250 µl contained the microsomal fraction (0.14–0.23 mg protein), Tris-HCl (0.1 M; pH 7.4), EDTA (40 µM), magnesium chloride (10 mM), UDPGA (2 mM), and 4-NP (500 µM), PPh (120 µM) or 4-MU (500 µM), except that with 4-MU, EDTA was omitted from the medium. Glucuronidation was measured on microsomes activated by the nonionic surfactant Lubrol-17A. The optimal mass ratio surfactant/protein was 0.25. The microsome-surfactant mixture was preincubated at 4 °C for 30 min. The glucuronidation was started by addition of UDPGA (0.01 M; 50 µl). The enzyme reaction was carried out at 37 °C for 10 min for 4-NP and PPh, and for 15 min for 4-MU. The transferase activity for 4-NP and PPh was measured colorimetrically at 405 nm and 550 nm, respectively. The fluorescence of 4-methylumbelliferyl-β-D-glucuronide was measured at excitation and emission wavelengths of 320 and 380 nm, respectively, with authentic 4-MU-β-D-glucuronide as a standard. The transferase activity was expressed as nmoles of glucuronide formed per min per mg protein. All herbicides tested were colourless and were added to the incubation mixture dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the incubation mixture was 20 g litre^{-1}. The control specific activity of UGT towards 4-NP was 20.9 ($\pm$2.13) nmol min^{-1} mg^{-1}. For the glucuronidation of PPh and 4-MU these values were 2.56 ($\pm$0.35) nmol min^{-1} mg^{-1} and 95 ($\pm$8) nmol min^{-1} mg^{-1}, respectively.

The apparent K_i values were determined from Dixon plots of the data (1/*v* versus [I]) varying the inhibitor concentrations between 0.5 and 1.5 mM.

3 RESULTS AND DISCUSSION

Twelve herbicides, representatives of two chemical groups (Table 1), were tested in our study for their inhibitory effect on the glucuronidation of three substrates: 4-nitrophenol, phenolphthalein and 4-methylumbelliferone, in rat liver microsomes. The results, expressed as percentages of inhibition of enzyme activities measured in the absence of herbicides, are given in Table 2. The main representative of the substituted phenoxyalkanoic acids, 2,4-D, as well

Table 1. List of herbicides tested

Common name	*Chemical name*
Substituted phenoxyalkanoic acid derivates	
1. 2,4-D	(2,4-Dichlorophenoxy)acetic acid
2. MCPA	(4-Chloro-2-methylphenoxy)acetic acid
3. Dichlorprop	(*RS*)-2-(2,4-Dichlorophenoxy)propionic acid
4. Mecoprop	(*RS*)-2-(4-Chloro-*o*-tolyloxy)propionic acid
5. 4-CPA	(4-Chlorophenoxy)acetic acid
s-Triazine derivatives	
6. Atrazine	6-Chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine
7. Propazine	6-Chloro-N^2,N^4-di-isopropyl-1,3,5-triazine-2,4-diamine
8. Cyanazine	2-(4-Chloro-6-ethylamino-1,3,5-triazin-2-ylamino)2-methylpropionitrile
9. Ametryn	N^2-Ethyl-N^4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine
10. Prometryn	N^2,N^4-Di-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine
11. Desethylatrazine	6-Chloro-N^4-isopropyl-1,3,5-triazine-2,4-diamine
12. Desisopropylatrazine	6-Chloro-N^2-ethyl-1,3,5-triazine-2,4-diamine

Table 2. Inhibitory effects of herbicides on UGT activities in rat liver microsomes

Common name	Concentration (mM)	Inhibition (%) (±SD)[a] 4-NP-UGT	PPh-UGT	4-MU-UGT
Substituted phenoxyalkanoic acid derivatives				
1. 2,4-D	1	12 (±2)	22 (±3)	20 (±2)
2. MCPA	0.1	–	*	–
	0.5	–	30 (±4)	–
	1	14 (±1)	48 (±5)	22 (±2)
3. Dichlorprop	0.1	13 (±1)	27 (±2)	17 (±2)
	0.5	22 (±1)	35 (±4)	26 (±4)
	1	30 (±4)	42 (±3)	35 (±3)
4. Mecoprop	0.1	*[b]	18 (±3)	10 (±1)
	0.5	21 (±2)	28 (±2)	21 (±2)
	1	34 (±7)	36 (±3)	32 (±1)
5. 4-CPA	1	*	12 (±1)	23 (±1)
s-Triazine derivatives				
6. Atrazine	1	0	18 (±2)	*
7. Propazine	0.1	11 (±3)	0	–
	0.5	22 (±1)	18 (±2)	–
	1	61 (±8)	36 (±1)	19 (±2)
8. Cyanazine	0.1	–	28 (±3)	17 (±2)
	0.5	–	40 (±3)	29 (±3)
	1	*	60 (±5)	36 (±1)
9. Ametryn	0.1	–	17 (±1)	0
	0.5	–	37 (±3)	14 (±1)
	1	27 (±3)	60 (±5)	36 (±1)
10. Prometryn	0.1	–	15 (±1)	*
	0.5	–	20 (±2)	10 (±1)
	1	0	35 (±2)	25 (±4)
11. Desethylatrazine	1	16 (±1)	25 (±3)	28 (±3)
12. Desisopropylatrazine	0.1	–	–	17 (±1)
	1	28 (±2)	18 (±2)	28 (±3)

[a] Mean values (±SD) of at least three experiments.
[b] Less than 10% inhibition.

as its dechlorinated derivative 4-CPA were weakly inhibitory against the enzyme activities studied. Replacement of the chlorine atom at the second position of the phenyl ring by a methyl group (MCPA) did not contribute much to the inhibitory potency of this herbicide against 4-NP and 4-MU glucuronidation. At 1 mM concentration, however, MCPA exerted a significant inhibitory effect on the PPh-converting isoenzymes (about 50% inhibition). MCPA is a hormone-type herbicide, extensively used for post-emergence control of annual and perennial broad-leaved weeds. In Canada, MCPA has been detected at relatively high levels in some ground-waters (1.0 mg $litre^{-1}$).[21] MCPA acute oral LD_{50} value for mice was found to be 500 mg kg^{-1}.[22] On immortalized cell cultures, MCPA was shown to exert its primary effect on mitochondria, increasing the mitochondrial membrane potential and inhibiting the ATP-synthesizing ATPase.[23] Agricultural exposure of farmers to 2,4-D and MCPA formulations has been reported to result in short-term immunosuppressive effects.[7]

The derivatives of 2,4-D, dichlorprop and mecoprop, were somewhat less effective against PPh glucuronidation than MCPA, exhibiting nearly the same effect also towards conjugation of the other two substrates. Thus, it may be suggested that UGT isoforms converting 4-NP, PPh or 4-MU show no difference in their sensitivity to these substances. It should be noted, however, that 0.1 mM concentration of dichlorprop was still inhibitory against PPh-glucuronidation (27% inhibition). There are practically no data in the literature about the biological sites of action of dichlorprop and mecoprop in animal cells. Acute oral LD_{50} values of these herbicides for mice are reported to be 400 mg kg^{-1} for dichlorprop and 650 mg kg^{-1} for mecoprop.[22]

Unlike 2,4-D, mecoprop and MCPA are methyl-substituted phenoxyalkanoic acids, with mecoprop and dichlorprop bearing one methyl group in the propionic acid residue. Obviously, the presence of methyl groups in the structures of these compounds contributes to their higher inhibitory potency in comparison with 2,4-D.

The next group of the tested herbicides included representatives of *s*-triazines. Triazine herbicides are widely used in agriculture, so that the risk of exposure to these chemicals is very high. Recently, certain triazine herbicides, primarily the chlorotriazines, have been reported to cause an increased incidence of mammary tumours in female Sprague–Dawley rats.[3]

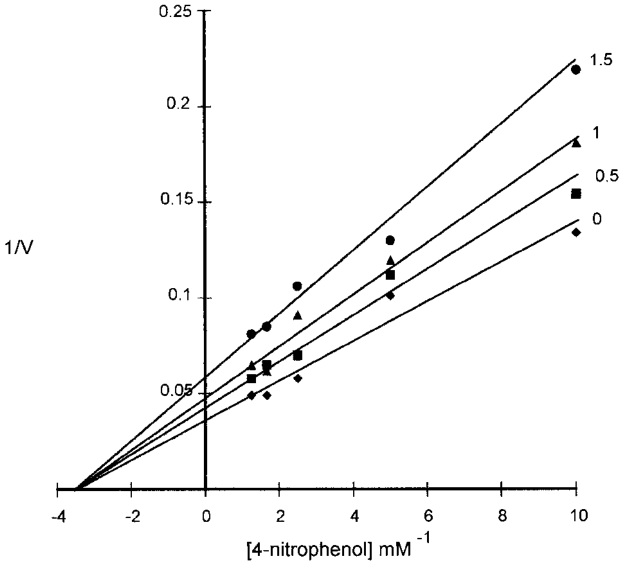

Figure 1. Lineweaver–Burk plot for the inhibition of 4-NP glucuronidation in rat liver microsomes by propazine. The 4-NP glucuronidation was assayed at varying concentrations of the aglycone in the presence of different concentrations of propazine. Herbicide concentrations (mM) are indicated on the right side of the plot.

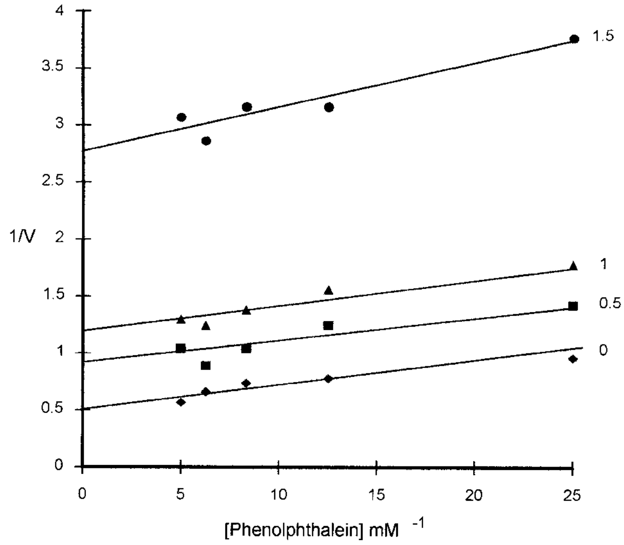

Figure 2. Lineweaver–Burk plot for the inhibition of PPh glucuronidation in rat liver microsomes by ametryn. The assay was carried out at varying concentrations of PPh in the presence of different concentrations of ametryn. Herbicide concentrations (mM) are indicated on the right side of the plot.

In previous work, the influence of two main representatives of these herbicides, simazine and atrazine, on UGT in rat liver microsomes has been reported.[15] Here we have extended our study, including four more differently substituted *s*-triazines as well as two atrazine metabolites. Atrazine, which was previously found to be fully inactive against the glucuronidation of 4-NP, exhibited only a marginal inhibitory activity towards PPh conversion. Another chlorotriazine herbicide, propazine, in which the ethyl group is replaced by a second isopropyl, was a much more effective inhibitor of the UGT activities studied than atrazine. At 1 mM concentration, propazine was found to be a strong inhibitor of 4-NP glucuronidation (61% inhibition), exhibiting a moderate activity against PPh conversion, and affecting to a lower extent 4-MU conjugation. Kinetic studies with this herbicide revealed a non-competitive type of inhibition towards 4-NP, with an apparent K_i value of 0.540 mM (Fig 1). Propazine has been reported to be non-toxic to mammals. There are very few data concerning its side effects in animal cells. Its acute oral LD_{50} value for rats amounted to more than 7000 mg kg^{-1}.[22]

The chlorotriazine herbicide cyanazine affected selectively the UGT activities. Like atrazine, it was inactive towards 4-NP glucuronidation. However, a strong inhibition of PPh conversion was shown. Even a 0.1 mM cyanazine concentration proved to be inhibitory against PPh glucuronidation (28% inhibition). 4-MU conjugation was decreased by about 36% in the presence of 1 mM cyanazine. This herbicide has a similar chemical structure to atrazine, bearing one additional cyano group. Obviously, this group is responsible for its higher inhibitory potency against UGT isoforms converting PPh and 4-MU, compared to atrazine. Cyanazine acute oral LD_{50} doses were reported for mice to be 380 mg kg^{-1}, and, for rabbits, 141 mg kg^{-1}. In rats and dogs, following oral administration, cyanazine was rapidly metabolized and eliminated within four days.[22]

The major metabolites of atrazine, desethylatrazine and desisopropylatrazine, were tested in this study. Removal of the ethyl or isopropyl group of atrazine did not lead to a higher inhibitory potency. A low decrease (by 16–25%) of UGT activities was registered in the presence of both metabolites.

Two sulfur-containing triazine herbicides, ametryn and prometryn, were also examined. Ametryn differs from atrazine by having a methylthio group as replacement for the chlorine atom. This substitution proved to be highly effective for the inhibitory potency of ametryn, especially against UGT activities associated with the conversion of PPh. Unlike atrazine, ametryn exhibited a strong, selective inhibition of PPh glucuronidation. To further characterize the interactions of this compound with UGT isoforms converting PPh, kinetic studies were conducted by varying both the concentration of PPh and UDPGA using inhibitor concentrations between 0.5 and 1.5 mM. An uncompetitive type of inhibition was observed with respect to PPh (Fig 2), and a mixed type of inhibition towards UDPGA (Fig 3), with apparent K_i values of 0.330 mM and 0.380 mM, respectively. The mixed type of inhibition suggests that ametryn competes, at least in part, for the binding site of UDPGA. Concerning the other two substrates, 4-NP and 4-MU, ametryn was nearly two times less effective against their conjugation.

Ametryn is a commonly used, synthetic herbicide. Studies of ametryn residues in fresh-water fishes in different ponds in Egypt revealed mean levels of 36–

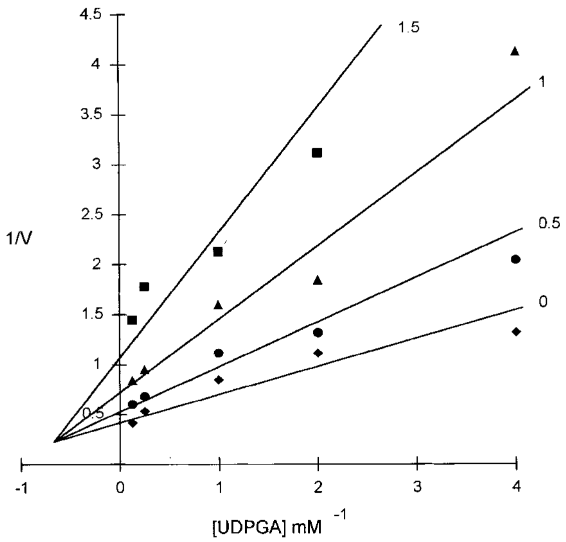

Figure 3. Lineweaver–Burk plot for the inhibition of PPh glucuronidation in rat liver microsomes by ametryn. The assay was carried out at varying concentrations of UDPGA in the presence of different concentrations of ametryn. Herbicide concentrations (mM) are indicated on the right side of the plot.

43 ng ametryn g^{-1} fish flesh.[5] Inoculation of sublethal doses of 1 and 2 mg kg^{-1} ametryn (0.01 mM) in to common carp (*Cyprius carpio* L) for four weeks led to a decrease in total erythrocyte and leukocyte count, hemoglobin concentration and total protein.[24] ATPase activity of silkworm larvae was found to be inhibited by ametryn doses equal to LD_{50} or even 1/10 LD_{50} values.[4]

Prometryn was less inhibitory than ametryn. It did not affect 4-NP glucuronidation and, compared to ametryn, exhibited two times less inhibitory activity against PPh or 4-MU conversion. Doses of 0.465 to 1.392 μmol prometryn have been reported to produce a significant in-vitro inhibition of enzyme activities responsible for testosterone conversion in the rat prostate.[1]

4 CONCLUSIONS

The results presented in this study reveal that more than the half of the tested herbicides exhibited a significant inhibitory effect on PPh-UGT activity in rat liver microsomes. As a whole, *s*-triazine herbicides were more inhibitory than the substituted phenoxy-alkanoic acids. Propazine, ametryn and cyanazine were found to be the most potent inhibitors of 4-NP-UGT (propazine) or PPh-UGT (ametryn, cyanazine) activities. The inhibition of 4-MU glucuronidation by the agrochemicals was relatively low and not specific. It should be mentioned that the tested 1 mM herbicide concentration producing an in-vitro enzyme inhibition exceeds by far that which would be expected in tissues of animals exposed to these chemicals. However, 0.1 mM concentrations of dichlorprop and cyanazine were still inhibitory against PPh conversion. It is not known if inhibitory effects on UGT would be expressed *in vivo* at 0.1 mM concentration of these compounds. The literature data on the accumulation of pesticides in animal tissues are scarce. Our findings might be useful when evaluating the toxicological properties of these herbicides in animal cells.

ACKNOWLEDGEMENTS

This work was supported by Grant No B-624 of the National Science Fund at the Bulgarian Ministry of Education, Science and Technology.

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